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IN VIVO INTESTINAL ABSORPTION OF SUGAR IN THE TOADFISH (MARINE TELEOST, *OPSANUS TAU*)

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Previous studies on the intestinal absorption and transport of nutrients in fish have been generally restricted to *in vitro* experiments. In such investigations everted or uneverted sacs or segments of intestinal tissue have been used to examine the mechanisms of sugar, amino acid, ion, and water transport (Aull, 1966; Carlisky and Huang, 1962; House and Green, 1965; Huang and Rout, 1967; Huang and Chen, 1971; Mepham and Smith, 1966; Musacchia, Westhoff and Van Haaren, 1966; Neff and Musacchia, 1967; Read, 1967; Rout, Lin and Huang, 1965; Sharratt, Bellamy and Jones, 1964; M. W. Smith, 1966, 1967; R. L. Smith, 1969; Stokes and Fromm, 1964; and Wilson, 1957).

The role of the intestine in ionic and osmotic regulation of fish has usually been examined under well defined *in vivo* conditions (Dall and Milward, 1969; Evans, 1967; Maetz, 1970; Potts, Foster, Rudy and Howell, 1967; Potts and Evans, 1967; Skadahauge, 1969; and Shehadeh and Gordon, 1969). However, *in vivo* investigations of the intestinal absorption of sugars and amino acids in fish have been limited to the early experiments of Cordier and his associates (Cordier and Channel, 1953; and Cordier, Maurice and Worbe, 1957) and Buclon and Peres (1963). Unfortunately these studies were not executed under rigorously defined conditions. For example, net transfer of water from the lumen was not measured. Since the intestinal absorption of water is appreciable when fish are kept in sea water, it can substantially influence the measurements of net solute transfer in the intestine (Maetz, 1970; Potts and Evans, 1967; and Skadahauge, 1969). Other objections to the earlier experiments cited include: (1) Sugar and amino acid solutions directly introduced into the intestine were not saline solutions and their osmolarities were often unknown. (2) The concentrations of substrates were unusually high (Cordier, Maurice and Worbe, 1957) and the time course of the experiments arbitrarily long. (3) The absorptive region of the intestine could not be defined and controlled due to the inadequacy of the technique employed by these investigators.

The present paper describes a rigorously defined technique for the study of intestinal absorption of nutrients in fish under *in vivo* free swimming conditions. This procedure is not only free of the above mentioned deficiencies but will permit rather precise measurements of the absorption rates of various nutrients under physiological conditions closely approximating those in nature. Consideration of natural physiological conditions are particularly important in cases where *in vitro* studies have led to enigmatic results. For example, flounder, trout, and toadfish maintain blood sugar levels which may be higher than 30 mg%, yet attempts to show active transport of sugars by *in vitro* intestinal segments of

these fish have failed (Nace, Monle and Schuh, 1963; Rout, Lin and Huang, 1965; Stokes and Fromm, 1964; and Wilson, 1957).

This *in vivo* approach also permits a new evaluation of the "sodium-coupled transport" mechanism proposed by several investigators (see Crane, 1968; Fordtran and Rector, 1971; and Schultz and Curran, 1970). Preliminary reports of this work have already been presented (Farmanfarmaian, 1971 and Farmanfarmaian, Ross and Mazal, 1971). The details will be reported in another communication.

Methods and Materials

Animal collection

Specimens of toadfish (*Opsanus tau*), weighing 150–380 g, were obtained from the Supply Department of the Marine Biological Laboratory (MBL). These animals were collected by local fishermen in the shallow waters of lower Cape Cod in the spring.

The collection procedure is based upon the mating and brooding habits of the toadfish. Empty cans are placed in shallow bays in the spring. During May and June paired toadfish choose these cans as nest sites. The female lays the eggs in the nest and departs while the male remains in or near the nest to guard it until the young leave (Gray and Winn, 1961). The fishermen collect the cans during the guarding period which produces only spent males. If during the same period baited traps are used, the collection will produce mainly spent females since the males do not wander far from the nest. During late summer and fall baited trap collections usually produce a mix of both sexes in various stages of gonad development. The latter have enlarged livers indicating active feeding and storage during the summer.

In this project toadfish were used in all seasons. Repetition of baseline measurements of glucose absorption rate showed no significant ($P < 0.05$) difference between sexes, seasons, or conditions of gonad and liver. Nonetheless, baseline rates were repeated for each new collection of fish.

Animal maintenance

The animals were initially maintained in the large running sea water tanks of the MBL Supply Department at approximately 15° C and were fed live killifish (*Fundulus*) *ad libitum*.

From these stock tanks, healthy animals were transfered to smaller laboratory tanks and acclimated at 20° C ± 1 for about one week. The feeding regimen was as above but each fish was under close observation during this period. All fish that exhibited abnormal feeding or swimming behavior or showed any external wounds and infections were eliminated.

Finally, the fish were starved for approximately 48 hours before an experiment.

The animals used during fall and winter at Rutgers University were shipped from the MBL Supply Department holding tanks by bus or car. They usually arrived in good condition after 6–7 hours of transit in well oxygenated sea water of 5–15° C. In the laboratory 20 animals were placed in each 35 gallon holding tank. The sea water was made from Rila Marine Mix (Rila Products, Teaneck,

New Jersey) and its final specific gravity adjusted to 1.025. Each holding tank was fitted with two large Dynaflow pump-filter systems (Metaframe Company, Maywood, New Jersey). Each of these units recirculated the sea water through the glasswool-activated-charcoal filter at a rate of about 4 liters per minute. Filters were changed several times a week as needed. In addition the tanks were aerated through cotton filters by means of separate air pumps. These tanks were in a cold room at 15° C ± 1 and on a 12 hour light-dark cycle. The toadfish were maintained under the same diet regimen and observation indicated above. Live trout fingerlings were used as food during the winter when specimens of *Fundulus* were not available. The animals were then acclimated for about one week at 20° C ± 1. Other conditions were similar to those described above. These toadfish were also starved for about 48 hours before surgery.

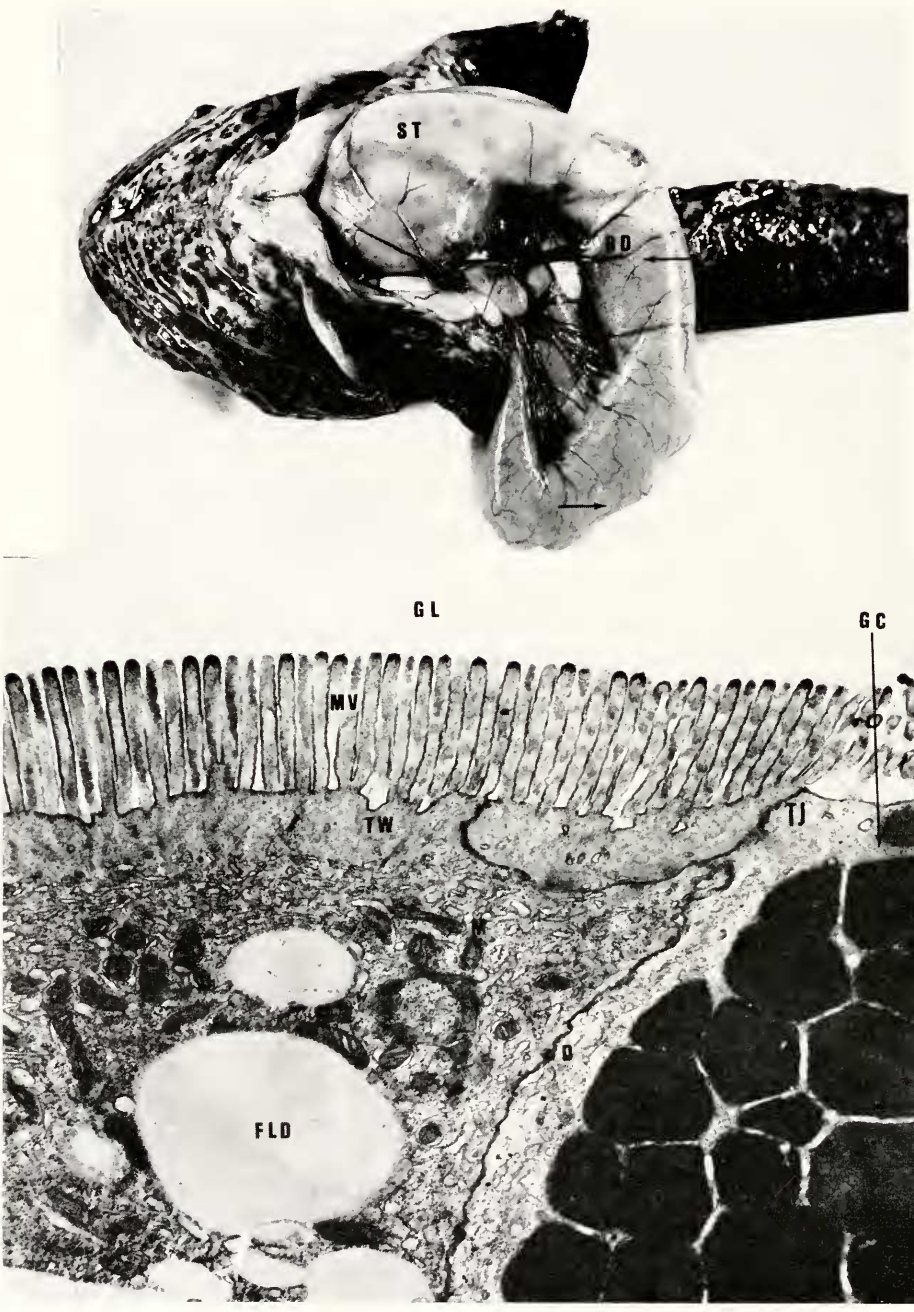
In all cases experiments were performed in healthy animals which had empty stomachs and upper intestines but showed gray or white fecal matter in the lower part of the gut. Unless otherwise indicated, all the experiments were carried out at 20° ± 1.

Surgical operations

The experimental fish was transfered from the starvation tank into a plastic bucket containing a "knock-out" anesthetic solution. This solution was prepared by dissolving 0.5 g of MS-222 (ethyl m-aminobenzoate methanesulfonate, Sigma) in a liter of sea water. When the opercular movements became reduced and the animal could not right itself, it was removed, drained on paper towel, and weighed. The toadfish was then placed upon a sponge covered V-shaped fish rack in a supine position. The rack was in a plastic pan containing a maintenance anesthetic solution (0.1 g MS-222/liter sea water). The solution was siphoned into a Dynaflow pump chamber where it was saturated with oxygen and then pumped through a Y-tube placed in the mouth of the fish over the gill arches. The solution returned to the pan by gravity flow. The pump maintained a flow of about 1 liter minute over the gills. Toadfish could be kept under anesthesia in this manner for several hours without harm. The heart beat could be visually observed or palpated ventrally just anterior to the pectoral girdle.

When the fish showed no movements other than occasional opercular contractions, an incision was made 2–3 cm anterior to the anus and extended to within one cm of the pectoral girdle. The body walls were retracted and the viscera exposed. These operations resulted in little or no bleeding if the iliac vessels near the anal region remained intact.

The intestine (midgut) was brought into full view and the common bile duct was located. The position of the bile duct in a well fed animal is illustrated in Figure 1. A 2 mm incision was made in the wall of the intestine at a point opposite to and just below the entrance of the bile duct (Fig. 1, anterior arrow). A tightly capped trocar was placed into the incision and its tip maneuvered in the posterior direction into the lumen. A ligature secured the trocar in place and simultaneously closed the intestine just below the entrance of the bile duct. At the lower extremity of the intestine a half centimeter incision was made and a large glass cannula, attached to a drainage tubing, was inserted into this slit. The lumen was washed by delivering three 5 ml portions of the desired saline



FIGURES 1–2.

in a syringe through the trocar. The wash fluid was drained out by gently lifting the gut. Finally, a second ligature was used to tie the posterior part of the experimental *in situ* gut loop at 6–7 cm below the trocar (Fig. 1, posterior arrow). The blood vessels were carefully avoided in these operations so that there was no bleeding and the circulation to the loop was intact. Furthermore such isolated loops were observed to undergo tonic and rhythmic contractions.

The desired test solution was directly intubated into the lumen of the loop through the trocar and the volume was adjusted so that the gut would be fully extended but exhibit little or no positive hydrostatic pressure. This volume was usually 4–5 ml. The loop was then gently shifted back and forth to mix and equilibrate the luminal contents rapidly. The trocar was opened and an initial sample of about 0.4–0.8 ml was drawn into a 1 ml syringe (graduated in 0.01 ml units) and the cap replaced. This sampling marked the starting time of the absorption period. The retractors were then removed and the abdomen rapidly closed with 9 mm Michel clips (Clay-Adams). The total surgical time was 7 ± 1 minutes. The fish was transferred to an oxygenated sea water tank and moved about to provide flow of sea water through the mouth over the gill arches. This resuscitation was maintained for a minute at which time the animal usually showed signs of revival (opercular and tail movements). In a few cases resuscitation extended over 2–4 minutes. The revived fish swam quietly in this tank and in all respects appeared normal (such animals could be kept alive for 8 days). Twenty-five minutes after the initial sample was taken, the fish was again transferred to the "knock-out" anesthetic solution. After complete anesthesia, the animal was returned to the fish rack and the abdomen rapidly opened. A 1.0 ml terminal sample was removed through the trocar and the time noted. The "incubation" or absorption period was 30 ± 1 minutes unless otherwise indicated. The experimental loop was excised by transversely cutting beyond the ligatures and stripping off the mesenteries. The external fluids were wiped off with tissue paper and the loop was inspected for leaks. Next the lower ligature was cut off over a wide mouth graduated centrifuge tube. The remaining luminal fluid was thus collected and its volume was recorded. The upper ligature and the tissue beyond it were trimmed off and the remaining true absorptive tissue was drained upon Whatman No. 1 filter paper. The wet weight of this tissue was determined on a Mettler H-10T balance. The tissue weights ranged between 1.5–3.5 g. Then the tissue was transfered either to iced 10% TCA for further analyses or discarded. The animal was sacrificed by excision of the heart under anesthesia.

FIGURE 1. Illustration of viscera in a freshly dissected fed toadfish. This animal weighed 198 g and contained 39 g of *Fundulus* which had been made available during the preceding 24 hours. The photograph shows the food in the stomach (ST) and the upper extremity of the intestine. Note the bile duct (BD) which serves as an anatomical marker for the insertion of the trocar. The arrows mark the limits of experimental loop. This region of the intestine is particularly marked by extensive circulation. Magnification is $\frac{1}{2}$ of natural size.

FIGURE 2. Electron micrograph showing the microvilli (MV) of an absorptive epithelial cell extending into the gut lumen (GL). Tight junctions (TJ) can be observed in the region of the terminal web (TW) where neighboring epithelial or goblet cell (GC) adhere together. Below the terminal web area mitochondria (M) and free lipid droplets (FLD) may be observed. The electron micrograph was kindly provided by Dr. M. L. Cayer. Magnification is 14,000 ×.

Solutions

The physiological salt solution used in these investigations was similar to the marine teleost Ringer developed by Forster and Taggart (1950) and therefore will be referred to as "FTR." The final millimolar concentration of the solutes in this saline were: NaCl, 134; KCl, 2.5; $CaCl_2 \cdot 2H_2O$, 1.5; $MgCl_2 \cdot 6H_2O$, 1.0; NaH_2PO_4, 0.5; and $NaHCO_3$, 15.0. The osmolarity of the FTR is approximately 280 milliosmoles per liter. This saline was gassed with 95% O_2–5% CO_2 before use in order to oxygenate and stabilize the pH at 7.2–7.3.

Test solutions containing D-glucose, inulin or other solutes were prepared in FTR. Tritiated or "cold" inulin (5000 MW, New England Nuclear Corp. and Fisher Scientific Company, respectively) was used to measure fluxes of water and the tissue extracellular space. The ratio of the terminal concentration to the initial concentration of inulin was used to correct changes in glucose concentration which resulted from changes in lumen water volume.

Chemical analyses

Glucose was measured colorimetrically by a highly purified glucose oxidase (Glucostat Special, Worthington Biochemical Corp.). The reagent was prepared in 0.2 M pH 7.0 Tris buffer (Sigma Chemical Co.). The procedures were similar to those described in earlier papers (Farmanfarmaian, 1969a and 1969b).

Inulin was colorimetrically determined by a modification of the method of Heyrovsky (1956). It was found that the purple violet color of this 3-indoleacetic acid reaction is stable at 530 nm for an hour if the reaction tubes are incubated for 80 minutes at 37° C and then equilibrated for 20 minutes at room temperature.

The water content of intestinal tissue was determined from the difference in the weight of the drained fresh tissue and its constant dry weight.

Radioisotope procedures

All radiochemicals were purchased from New England Nuclear Corporation. Radioactivity was measured by means of a Packard or a Picker liquid scintillation counter. The procedures for these measurements as well as thin layer chromatographic identification of labeled compounds was described previously (Farmanfarmaian, 1969b).

Histological preparation

Mucosal tissues from the experimental region of the intestine were fixed in 3% glutaraldehyde phosphate buffer at pH 7.0. This was followed by post-fixation in 1% osmium tetroxide-phosphate buffer pH 7.0. The tissues were then dehydrated in acetone, embedded in Spurr's medium and examined on a Hitachi-8-1 electron microscope.

Treatment of data

Data are reported as mean $\pm$ the standard error at $P \leq 0.05$ (N) unless otherwise indicated. Computations related to the radiotracer method were in accordance with Wang and Willis (1965). Statistical treatment of the data was

in accordance with procedures found in Mather (1951). Unless otherwise specified absorption or transport rates are expressed in units of μmoles/g fresh intestinal tissue/hour.

Results

Anatomical and histological observations

In the toadfish the gastrointestinal tract consists of a short esophagus extending from the oropharyngeal sphincter to a thick walled stomach which is slightly J-shaped (Fig. 1). The small intestine or midgut extends from the pyloric constriction to the short hindgut near the anus. The intestine is essentially a straight tube, lacking any of the diverticulae present in other teleosts. It is 1–3 cm in diameter and 12–18 cm in length. It receives its blood supply mainly from the mesenteric artery and is drained by the hepatic portal vein.

Table I

Effect of MS-222 anesthesia on the intestinal absorption of D-Glucose in the toadfish, Opsanus tau

	Initial glucose conc. mM	Absorption rate μmoles/g tissue/hour	Terminal intestinal tissue conc. μmoles/ml tissue H_2O
Anesthetized during absorption	10	6.83 ± 0.81 (11)*	5.72 ± 1.0 (11)*
Swimming during absorption	10	6.86 ± 1.17 (6)	3.42 ± 0.98 (6)

* values are means ± SE (N) at $P \leq 0.05$.

Histological sections examined by light microscopy were generally similar to those of mammals except that the folds of mucosa extending into the lumen were thicker and more extensive (Bloom and Fawcett, 1968). The microanatomy of the mucosal epithelium was examined by electron microscopy. The absorptive epithelial cell is about 20 μ in height and has an average diameter of 5 μ. Its luminal border is covered with microvilli which are about 1 μ long and 0.1 μ in diameter (Fig. 2). At a magnification of 50,000 × delicate filaments covering the microvilli are discernible. These have been previously observed on cat microvilli and referred to as "fuzz" by Ito (1965). In the area of the terminal web at the sides of the cells the outer membranes form a junctional complex including tight junction, intermediate junction, and desmosomes. Below the terminal web other cell organelles, such as lysosomes, mitochondria, reticulum, and nucleus, much like those described for mammals, can be seen.

In summary, the toadfish intestinal mucosa is covered by an absorptive epithelial cell layer which exhibits anatomical features similar to those observed in the well known absorptive epithelium of the mammalian intestine.

Validation of methodology

A number of experiments were designed to test and establish the validity of the analytical and experimental procedures adopted here.

In a series of toadfish the intestinal loop was filled with FTR alone in order to test the effect of the saline on endogenous constituents such as water, glucose,

and substances which give a positive Heyrovsky reaction (see methods and materials section). After 30 minutes under free swimming conditions no glucose or Heyrovsky reactive material could be detected in the lumen fluid. Analysis of the tissue showed only a slight reaction to the Heyrovsky test but the mean

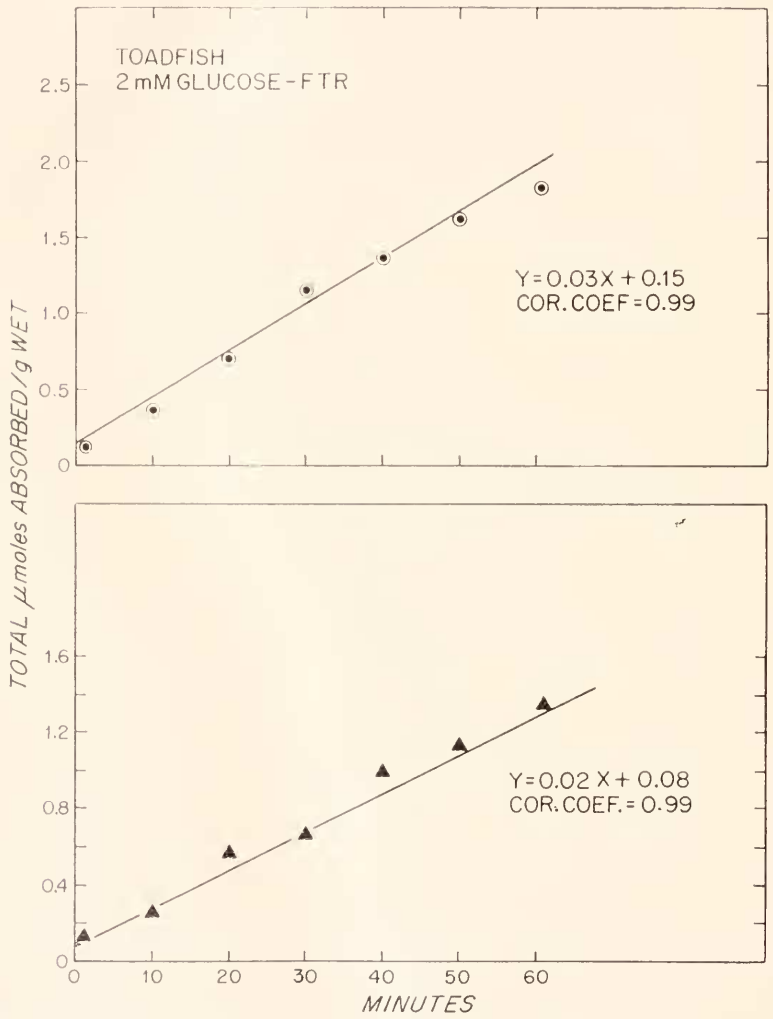


FIGURE 3. Time dependency of glucose absorption by toadfish intestine *in vivo*. Initial glucose concentration was 2 mM.

glucose concentration in micromoles per milliliter of tissue water was appreciable, 1.22 ± 0.13 (5). Tissue water expressed as per cent of standard wet weight was 84.6 ± 0.93 (13).

The effect of MS-222 anesthesia on the absorption of glucose was investigated in two groups of animals. The first group remained under maintenance anesthesia

on the rack while the second group was revived after the operation and allowed to swim during the 30 minute absorption period. In both groups a 10 mM glucose-FTR-^{3}H inulin solution was used. The results reported in Table I show that there is no significant difference in the glucose absorption rate of the two

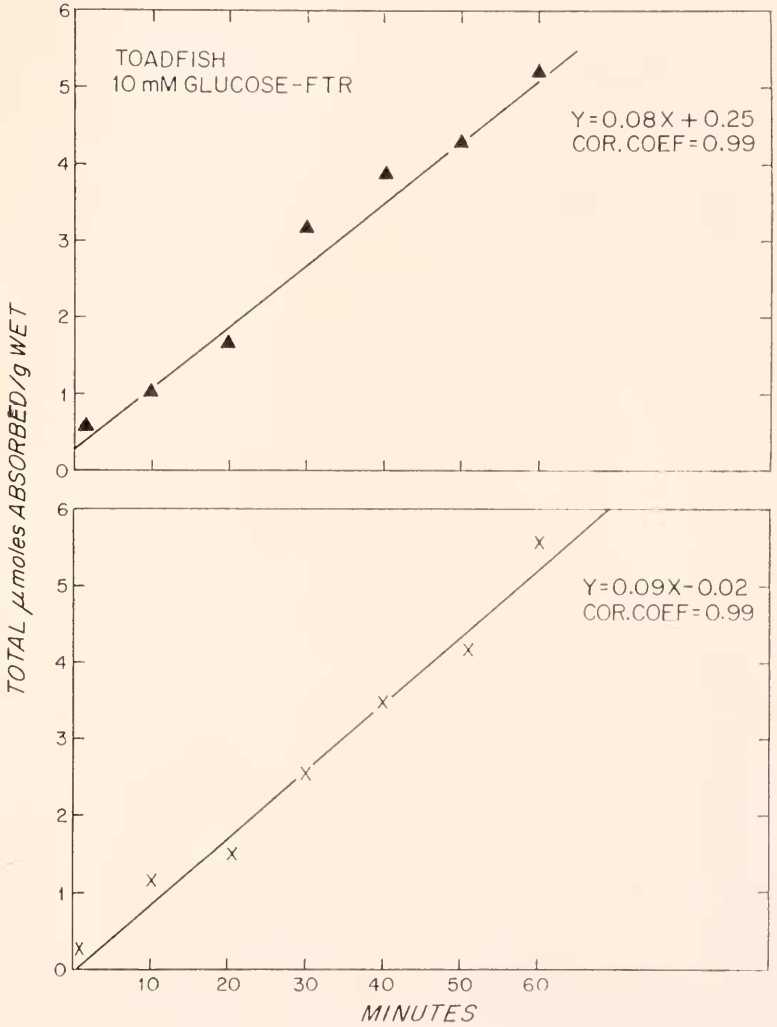


FIGURE 4. Time dependency of glucose absorption at 10 mM initial concentration.

groups. The terminal tissue concentration of the anesthetized group was higher. This is probably due to the supine position under anesthesia which places external pressure upon the veins draining the loop. The resulting reduction in circulation causes a reduced transfer of glucose from the interstitial fluid to the blood. Thus glucose tends to accumulate in the tissue.

Valid transport rate constants are most conveniently determined in experimental systems where there is a linear relation between the total absorption of substrate and time. Once this linearity is established, a practical time interval (*e.g.,* 30 minutes) may be chosen for kinetic studies. The time dependence of glucose absorption was therefore investigated at 2 and 10 mM concentrations. Samples were removed through the trocar at various time intervals while the fish was under maintenance anesthesia. In these experiments the initial volume of the intubated test solution was usually 5 ml and the sample size was reduced to approximately 0.2 ml in order to sustain a large mass of substrate in the lumen during the experiment. The experiment was repeated for each concentration in 4 or more fish. Typical results are depicted in Figures 3 and 4. The lines are fitted from the calculated regressions but the points are those obtained experimentally. The linear correlation coefficient was higher than 0.96 in all cases and the intercepts were close to zero. For all practical purposes the system

TABLE II

Absorption of D-Glucose in different regions of the intestine of the toadfish, Opsanus tau*

Fish wet weight (g)	Rate of absorption in μmoles/g tissue/hour		$\frac{\text{Lower} \times 100}{\text{Upper}}$
	Upper third	Lower third	
253	7.08	0.09	1.27
310	3.17	0.63	19.89
382	4.74	0.13	2.75
275	5.48	0.40	7.26
275	6.35	0.89	14.14

* Initial solution was 10 mM glucose-FTR. Both regions were tested in the same fish simultaneously under swimming conditions.

behaves like a zero order reaction within the chosen 30 minute limit for incubations. Departure from origin occurred when the test solution was not properly mixed within the lumen at the outset of the experiment.

Since different regions of the fish intestine may have different absorptive capacities, it was necessary to establish whether the anterior third of the small intestine chosen for our experiments is in fact the physiologically significant region of normal sugar absorption. The intestine of the toadfish is relatively short, therefore two comparable experimental loops were prepared within the same animal. The upper loop included the first 5 cm of the anterior extremity, and the lower loop consisted of the last 5 cm of the posterior extremity of the intestine. The absorption of glucose was simultaneously determined for both loops in free swimming fish. The results are presented in Table II. In every case the posterior third of the gut had a much lower capacity for glucose absorption in comparison with the anterior third.

Characterization of the glucose transport system

Solute transfer across cell membranes can proceed by one or more of three general processes. These are simple diffusion, facilitated diffusion, and

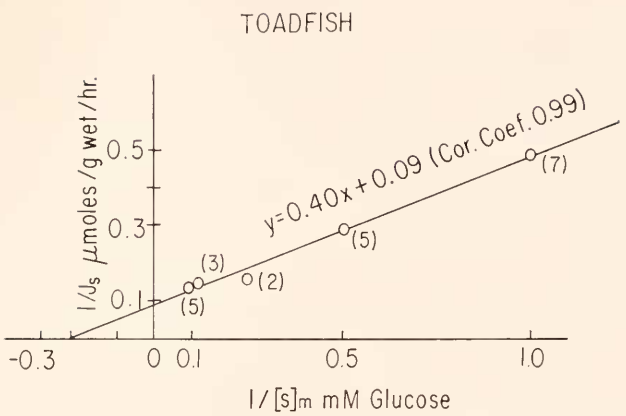


FIGURE 5. Double reciprocal plot showing the relation between net glucose absorption rate and glucose concentration. The line is fitted from the calculated regression but the points represent the actual mean for (N) fish at each concentration.

active transport. The last two are thought to be "carrier mediated." The definition, criteria for recognition, and experimental observation of these processes in various tissues have been reviewed by Stein (1967). The strongest criteria for "carrier mediated" transport under *in vivo* conditions appear to be (1) saturation kinetics and (2) sensitivity to specific inhibitors of the transport system. The phenomenon may be described as active transport if, in addition to the above criteria, it can be shown that the substrate is transported against a chemical (or electrochemical) gradient. We have used all three criteria to characterize the process of glucose absorption in the intestine of the swimming toadfish.

First, the absorption rate of glucose was determined as a function of substrate concentrations between 1 mM and 10 mM. As expected, the transfer of this sugar proved to be a saturation phenomenon conforming to the familiar Michaelis-Menten equation, $v = (Vmax)[S]/(Km + [S])$. There are three linear transformations of this equation (Dowd and Riggs, 1965). The data are plotted in Figure 5 in accordance with the following transformation using notations which are more suitable to transport studies: $1/J_s = (K_t/J^{max})(1/[S]_m) + (1/J_s^{max})$ where J_s denotes net flux of substrate S and J_s^{max} is the net maximum flux; $[S]_m$ is the mucosal concentration of S and K_t is the substrate concentration at which

TABLE III

Effect of 5 × 10⁻⁴ M phloridzin on the intestinal absorption of D-Glucose in the toadfish, Opsanus tau, *in vivo*

Initial glucose Conc. mM	Absorption rate	
	Control	Phloridzin
1.0	2.07 ± 0.2 (4)*	0.13 ± 0.1 (5)
10.0	6.86 ± 1.1 (6)	−0.16 ± 1.7 (4)

* Mean rates are given in µmoles glucose/g tissue/hour ± SE(N) at $P \leq 0.05$.

half net maximal flux is recorded. The other transformations were tested, but yielded no improvement in analysis or representation of the data. The transport constants K_t and J_s^{max} (and their 95% confidence limits) calculated from the regression of the fitted line were 3.7 (3.3–4.1) mM and 9.7 (8.4–11.7) μmoles/g tissue/hour.

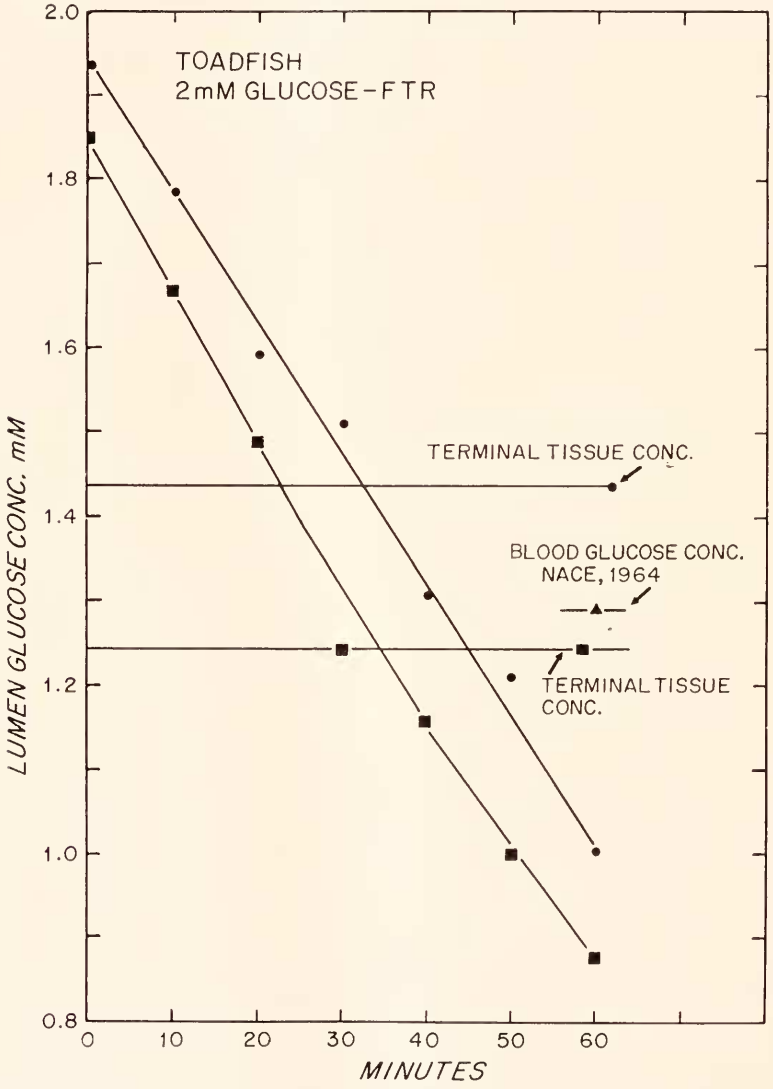


FIGURE 6. Reduction of glucose concentration in the lumen to a level below the tissue and blood glucose concentration due to active transport. The upper horizontal line ● labelled terminal tissue concentration refers to upper absorption curve. The lower horizontal line ■ indicates the terminal tissue concentration for the lower absorption curve. Blood glucose concentration ▲ indicates the mean blood glucose level reported by Nace (1964) for toadfish during the summer season.

Secondly, phloridzin, a well-known inhibitor of the sugar transport system (Malathi and Crane, 1969) was used to test for specific inhibition of this transport system. The results of experiments conducted at 1 mM and 10 mM glucose in presence of 5×10^{-4} M phloridzin are shown in Table III. At both concentration phloridzin abolished glucose absorption. For the higher concentration a slight rise in the luminal glucose was noted (as indicated by the negative absorption rate) in the phloridzin treated animals. Although the magnitude of this rise was not statistically significant ($P \leq 0.05$) in our experiments, the same observation has been reported by others and may prove to be real (Musacchia, Neff and Westhoff, 1964 and Smyth, 1971).

Finally, two types of experiments were done to determine whether intestinal transport of glucose in toadfish can proceed against a glucose concentration gradient under *in vivo* conditions. In contrast with *in vitro* preparations where substrate has a possibility of accumulating in the tissue or on the serosal side, under *in vivo* conditions absorbed substrate is rapidly carried away by blood. Therefore an uphill concentration gradient between the lumen and tissue cannot be observed

TABLE IV

Absorption and accumulation of D-Glucose in the intestine of the toadfish, Opsanus tau, in vivo

Initial glucose conc. mM	Absorption rate μmoles/g tissue/hour	Terminal intestinal tissue concentration μmoles/ml tissue H_2O	Terminal medium conc. mM	Terminal concentration tissue/medium
1.0	2.07 ± 0.21 (4)*	1.33 ± 0.36 (4)*	0.26 ± 0.16 (4)*	5.3
2.0	3.47 ± 0.62 (5)	1.59 ± 0.58 (5)	1.07 ± 0.22 (5)	1.48
10.0	6.86 ± 1.18 (6)	3.42 ± 0.98 (6)	8.03 ± 0.66 (6)	0.43

* Numbers are mean rates or concentrations ± SE (N) at $P \leq 0.05$.

until the luminal concentration is reduced below the tissue and blood concentration. Hence, *in vivo* experiments which are designed to show active transport should start with a low luminal concentration of the substrate (*e.g.,* twice the homeostatic blood concentration). These considerations are particularly applicable to an animal such as the toadfish in which the maximum velocity (J_s^{max}) for glucose absorption at 20° C is relatively low (9.7 μmoles/g tissue/hour). Accordingly, in the first series of experiments initial luminal glucose was set at 2 mM and absorption was followed at 10 minute intervals for 60 minutes under anesthesia. Tissue glucose concentration was determined immediately after the terminal sample. Typical results are plotted in Figure 6 for two fish. These results clearly show that glucose continues to be absorbed from the lumen solution after its concentration has fallen substantially below that of tissue and blood.

In the second series, the absorption rate, and the terminal lumen and tissue concentrations were measured for 1 mM, 2 mM, and 10 mM glucose solutions after a 30 minute interval in swimming fish. These measurements as well as the terminal tissue/medium concentration ratios are presented in Table IV. For the 1 mM and 2 mM initial concentrations the tissue/medium ratios are significantly greater than one. These results confirm the uphill transport of glucose against its concentration gradient and therefore the process can be called active

transport. Although absorption is high at 10 mM, the incubation time is not sufficient to reduce the concentration in the luminal medium below that of the tissue. The fact that phloridzin completely abolished glucose transport at 10 mM concentration (Table III) is a further indication that nearly all the glucose absorption at this concentration is also by an active transport process.

Discussion

A new method has been described here and applied to demonstrate the active transport of glucose in the intestine of free swimming toadfish. This method may be used to study the intestinal absorption of any nutrient or other substances in fish under well defined conditions approximating the normal physiological state of the animal in nature. The weaknesses inherent in previous *in vivo* investigations of sugar and amino acid transport in fish intestine have been rectified. In previous studies (Cordier and Chanel, 1951; Cordier and Maurice, 1956), the region of the intestine exposed to the nutrient solution could not be specified. These authors injected various solution via the anal pore and measured the absorption of the substrate after a specified time interval. When a solution is injected in this manner, it may pool in the posterior part of the intestine or spread throughout the gastrointestinal tract depending upon the fluid volume and the capacity of the gut. In the present study it has been shown that the rate of absorption of glucose in the lower third of the gut is about 10% that of the upper third of the intestine. Carlisky and Huang (1962) reported that glucose transport in segments of the "duodeno-jejunal" region of the dogfish was higher than the "ileum." Regional specialization of the intestine is also well established for the mammals (Booth, 1968). Therefore, *in vivo* absorption measurements cannot be considered valid unless the specific region of the intestine is clearly defined.

Another inadequacy of previous *in vivo* techniques was that the state of the intestinal contents, including food, fluid, parasites, and bacteria could not be ascertained prior to the experiments. Furthermore, samples could not be taken except at the end of the experiment. Since indicators such as inulin were not used to measure water volume, dilution by extant intestinal fluids erroneously appeared as reduction in substrate concentration. In the present technique the trocar allows for washing the loop of all its previous contents and for sampling at any desired interval. Inclusion of a water indicator was found to be essential since in most of the experiments there was an appreciable absorption of water from FTR solutions, amounting to 2–18% of the initial lumen volume.

The *in vivo* method permits the tonic and rhythmic contractions of the intestine to proceed normally. Therefore the problem of "unstirred layers," which may be a source of error under *in vitro* conditions is minimized. In our attempts to use isolated everted sacs of toadfish in glucose-FTR solutions the intestinal segments underwent severe sustained contractions. No transmural glucose transport could be observed in such preparations. These observations were similar to those reported for toadfish by Wilson (1957), for flounder by Rout, Lin and Huang (1965), and for trout by Stokes and Fromm (1964).

It is difficult to explain such a discrepancy in sugar transport between the *in vitro* and *in vivo* studies. However, any interpretation that rejects the occurrence of intestinal absorption of glucose in these and other fish is physiologically

untenable. Most fish have relatively high blood sugar and liver glycogen levels. Nace, Monle and Schuh (1963) reported that the blood glucose of toadfish was about 100 mg% between January and April and 20–30 mg% during the summer. Rout, Lin and Huang (1965) found the blood glucose concentration of the flounder to be between 26–50 mg%. Stimpson (1965) reported a liver glycogen value of 43 mg/g tissue in goldfish, nearly as high as that in rat (47 mg/g). It is unreasonable to suppose that such high levels of blood sugar and liver glycogen in fish, comparable to those of mammals in some cases, are entirely achieved through gluconeogenesis. In studies of the general intermediary metabolism of fish (Hochachka, 1969) there has been no evidence of unusually high gluconeogenetic activity. Absorption routes other than the intestine are unlikely; body and gill surfaces are not highly permeable to sugars and amino acids and the available quantities in the sea are not appreciable, 10^{-7} M (Siegel and Degen, 1966). An effective intestinal sugar transport system is indicated by the finding of efficient utilization of ingested carbohydrates in trout (Phillips, 1969). Therefore it is more reasonable to regard the intestine as the normal rout of sugar absorption and seek other explanations for the failure of its detection in some *in vitro* studies.

Wilson (1957) and Rout, Lin and Huang (1965) reported that intestinal segments of the toadfish and the flounder, which were capable of transmural uphill transport of amino acids, could not transport glucose in the same manner. An explanation for these observations may be found in the reduction of O_2 supply and the consequent Pasteur effect under *in vitro* conditions. Although flasks are meticulously gassed with 95% O_2–5% CO_2 in such experiments, the O_2 capacity of salines (about 0.5 volumes % at 20° C and less at higher temperatures) is a limiting factor. This capacity is adequate for tissues of some invertebrates, such as *Thyone* (Echinodermata), which normally maintain low O_2 tensions in their body fluids (Farmanfarmaian, 1966). But for most vertebrates the O_2 capacity of salines is at least one order of magnitude less than the O_2 capacity of their blood, usually between 6–20 volumes % (Prosser and Brown, 1961). Pietra and Cappelli (1970) have shown that the O_2 consumption of rat intestinal segments does not reach saturation even at pO_2 of 650 mm Hg, the highest pO_2 used. The stimulation of glycolysis, due to inadequate oxygen supply and Pasteur effect, increases the cellular utilization of glucose. For rat as much as 75% and for hamster 45% of absorbed glucose may be converted to lactate in isolated segments of the intestine (Wilson, 1956). In these mammals we find the *in vivo* and *in vitro* glucose absorption rate at 37° C to be several times higher than the swimming fish at 20° C (Farmanfarmaian, unpublished data). Therefore in the case of the mammalian intestinal segments the mass of absorbed glucose may be sufficient to permit mucosal intracellular accumulation and uphill transfer to the serosal side in spite of a high rate of glucose utilization under hypoxic conditions. In fish intestine, on the other hand, the glycolysis rate *in vitro* may be so high relative to absorption rate that the active transport to the serosal side is completely masked. Under these conditions, experimental limitations, such as sensitivity of glucose detection methods and tissue survival, become critical. Even in the rat it is difficult to demonstrate uphill transport of glucose *in vitro* unless the incubation period is extended beyond 30 minutes (Wiseman, 1964). Such extension is

eventually limited by desquamation and general tissue aging which reduce the absorption process.

The maximum velocity (J_s^{max}) and the concentration at which half maximal velocity is obtained (K_t) may be used as transport constants for a substrate which is transported by a "carrier-mediated" system conforming to Michaelis-Menten kinetics. These constants are of value for resolving components of transport mechanisms and for comparing data obtained under varying conditions or in different species. If the evolutionary unity observed in fundamental processes such as the genetic code, protein synthesis, and metabolic pathways is also true for the "carrier" mechanism of a widespread natural compound such as glucose, then one might expect the glucose K_t to be of the same order of magnitude in different species and under different physiological conditions.

These expectations are generally supported by the K_t values reported in the literature and our present investigations. When statistical variability for different studies is considered, K_t for the *absorption* of glucose by the intestinal mucosa and other tissues is within the same order of magnitude for a variety of vertebrates as well as invertebrates. In the present study K_t for the intestinal absorption of glucose in the swimming toadfish had a mean value of 3.7 mM (summer of 1970) and 4.1 mM (summer of 1971), the difference falling within the 95% confidence limits. For the hamster intestinal segments the reported K_t values are 1.5 and 2.5 mM (Crane, 1968). *In vitro* determinations of K_t for glucose absorption by sheets of rabbit ileum yielded a value of 5 mM when calculated from short circuit currents (Schultz and Zalusky, 1964) and 1.4 mM when measured as ^{14}C-glucose fluxes (Goldner, Schultz and Curran, 1969). In other mammalian tissues such as red blood cells and kidney slices the most frequent values reported for glucose absorption K_t (but not K_i) are between 1–5 mM (Stein, 1967). Kinetic studies of glucose absorption among invertebrates have been limited to the intestine of *Thyone* (Echinodermata), and the body wall of the rat tapeworm. In *Thyone* intestine, where glucose is absorbed by a facilitated diffusion process, the K_t measured with everted intestinal sacs in artificial sea water at 20° C was 1.6 mM (Farmanfarmaian, 1968). The K_t for body wall uptake by the rat tapeworm *in vitro* at 37° C is also 1.6 mM (Read, 1961).

In contrast to the narrow range for K_t, the reported values for maximum velocity (J_s^{max}) are highly variable. These data indicate that the binding site for glucose in the brush border membrane of the intestinal epithelium (and possibly other tissues) may be similar among species. On the other hand, the actual rates of transport which reflect number of functional sites (and possibly other factors) are highly dependent upon experimental conditions as well as the specific tissues and species. The relative constancy of K_t among organisms having different intestinal ionic concentrations in nature (*e.g.*, *Thyone*, toadfish, and rat) may shed light on the proposed role of Na^+ in sugar transport (Crane, 1968; Schultz and Curran, 1970; and Fordtran and Rector, 1971). The similarity in K_t values cited above would seem to indicate that the properties of the glucose binding site, as reflected by K_t are not significantly affected by changes in the extracellular Na^+, at least over the range occurring naturally. Since the possibility remains that the *luminal* concentration of Na^+ required for normal binding and transport of glucose is well below the level naturally available in the lumen, we have under-

taken a systematic *in vivo* investigation of this problem in several species. Our preliminary data indicate that a ten fold reduction in the *luminal* concentration of Na^+ in the swimming toadfish has no appreciable effect on K_t or J_s^{max} of glucose transport (Farmanfarmaian, Ross and Mazal, 1971). Details of this work will be published in a subsequent communication.

Messrs Ross and Mazal were Henry Rutgers honor students who made significant contributions to this research work in their senior year. The senior author is a member of the Bureau of Biological Research, Rutgers University.

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Summary

1. A technique for investigation of nutrient absorption *in vivo* in the intestine of free-swimming fish is described.

2. Glucose absorption occurs primarily in the upper third of toadfish intestine and is linear with time.

3. Anesthetized (MS-222) and free-swimming toadfish were similar with respect to intestinal absorption of glucose.

4. The absorption process exhibited saturation kinetics, conforming to the Michaelis-Menten equation. It is inhibited by phloridzin.

5. Active transport of glucose was demonstrated by the fact that absorption continues after the glucose concentration of the intestinal lumen has fallen below that of intestinal tissue and blood.

6. Difference in the *in vivo* and *in vitro* approaches to the study of intestinal transport and the significance of the observed kinetic parameters are discussed.

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